Suppression of γ-Aminobutyric Acid (GABA) Transaminases Induces Prominent GABA Accumulation, Dwarfism and Infertility in the Tomato (Solanum lycopersicum L.)

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journal or publication title	Plant and cell physiology
volume	54
number	5
page range	793-807
year	2013-02
権利	(C) The Author 2013. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.This is a pre-copyedited, author-produced PDF of an article accepted for publication in Plant and cell physiology following peer review. The version of record Plant Cell Physiol (2013) 54 (5): 793-807 is available online at: http://pcp.oxfordjournals.org/content/early/2013/04/12/pcp.pct035.
URL	http://hdl.handle.net/2241/00122684

doi: 10.1093/pcp/pct035

Running Title

SlGABA-T1 is responsible for GABA metabolism

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Subject Areas

(4) proteins, enzymes and metabolism

Number of black and white figures, color figures and tables

Seven black and white figures

Three color figures

One table

Title

Suppression of γ–aminobutyric acid (GABA) transaminases induces prominent GABA accumulation, dwarfism and infertility in the tomato (*Solanum lycopersicum* L.)

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Abbreviations.

CaMV: cauliflower mosaic virus, DAF: days after flowering, GABA: gamma-aminobutyric acid, GABA-T: GABA transaminase, GABA-TK: α-ketoglutarate-dependent GABA transaminase, GABA-TP: pyruvate-dependent GABA transaminase, GABA-TG: glyoxylate-dependent GABA transaminase, GABA-TP/TG: pyruvate- and glyoxylate-dependent GABA-T, GAD: glutamate decarboxylase, GHB: gamma-hydroxy butyric acid, MG: mature green, RNAi: RNA interference, RT-PCR: reverse transcription polymerase chain reaction, SSA: succinic semialdehyde, SSADH: succinic semialdehyde dehydrogenase, SSR: succinate semialdehyde reductase, TCA: tricarboxylic acid, WT: wild-type, Yell: yellow

Abstract

Tomatoes accumulate gamma-aminobutyric acid (GABA) at high levels in the immature fruits. GABA is rapidly converted to succinate during fruit ripening through the activities of GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH). Although three genes encoding GABA-T and both pyruvate- and α-ketoglutarate-dependent GABA-T activities have been detected in tomato fruits, the mechanism underlying the GABA-T-mediated conversion of GABA has not been fully understood. In this work, we conducted loss-of-function analyses utilising RNA interference (RNAi) transgenic plants with suppressed pyruvate- and glyoxylate-dependent GABA-T gene expression to clarify which GABA-T isoforms are essential for its function. The RNAi plants with suppressed *SlGABA-T* gene expression, particularly *SlGABA-T1*, showed severe dwarfism and infertility. *SlGABA-T1* expression was inversely associated with GABA levels in the fruit at the red-ripe stage. The GABA contents in *35S::SlGABA-T1*^{RNAi} lines were 1.3 to 2.0 times and 6.8 to 9.2 times higher in mature-green and red-ripe fruits, respectively, than the contents in WT fruits. In addition, *SlGABA-T1* expression was strongly suppressed in the GABA-accumulating lines. These results indicate that pyruvate- and glyoxylate-dependent GABA-T is the essential isoform for GABA metabolism in tomato plants and that *GABA-T1* primarily contributes to GABA reduction in the ripening fruits.

Keywords

GABA, GABA-TP/TG, tomato, fruit, dwarfism, infertility

Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid commonly found in bacteria, animals and plants. GABA is a major inhibitory neurotransmitter in vertebrates (Zhang and Jackson 1993) and has been identified as a functional component in reducing blood pressure in the human body (Takahashi et al. 1961, Inoue et al. 2003, Kajimoto et al. 2004). GABA is metabolised *via* a short pathway, called the "GABA shunt" (Fig. 1), which is a bypass of the tricarboxylic acid (TCA) cycle composed of three enzymes (Bouché and Fromm 2004, Shelp et al. 1999). In this shunt, GABA is irreversibly synthesised from glutamate through glutamate decarboxylase (GAD) (Chung et al. 1992, Ling et al. 1994) and reversibly converted to succinic semialdehyde (SSA) through GABA transaminase (GABA-T) (Shelp et al. 1999, Van Cauwenberghe et al. 2002). Succinic semialdehyde is subsequently irreversibly reduced through succinate semialdehyde dehydrogenase (SSADH) and eventually flows back into the TCA cycle.

In plants, GABA was first described in potato tubers in 1949 (Steward et al. 1949). Early studies showed that various environmental or non-environmental stresses, such as drought, UV irradiation, mechanical damage, low temperature and low O_2, promote GABA accumulation (Shelp et al. 1999, Snedden and Fromm 1999, Kinnersley and Turano 2000). Furthermore, GABA has been reported to function in the regulation of cytosolic pH (Bown and Shelp 1997), pollen tube growth (Palanivelu et al. 2003), the expression of nitrate transporter (Beuve et al. 2004) and cell elongation (Renault et al. 2011).

There have been many reports describing GABA synthesis, including the regulation and function of GAD, in various plant species (Chen et al. 1994, Rolin et al. 2000, Bouché et al. 2004, Akama et al. 2009). In contrast, in plants, knowledge on the conversion of GABA to succinate remains limited. Two isoforms of the GABA-T enzyme have been reported: pyruvate-dependent GABA-T (GABA-TP) and α-ketoglutarate-dependent GABA-T (GABA-TK) (Bouché and Fromm 2004). These enzymes utilise pyruvate or α-ketoglutarate as amino acid acceptors to produce alanine or glutamate, respectively (Bouché and Fromm 2004). GABA-TP has also

glyoxylate-dependent GABA-T (GABA-TG) activity, and utilizes glyoxylate as amino acids acceptor to produces glycine (Clark et al., 2009a; Clark et al., 2009b). However, although the activities of both enzymes have been detected in plants, such as soybean, tobacco, potato and tomato (Shelp et al. 1995, Van Cauwenberghe and Shelp 1999, Satya-Narayan and Nair 1986, Akihiro et al. 2008), the GABA-TK gene has not been identified in plants (Bouché and Fromm 2004).

The tomato accumulates high levels of GABA in its fruits (Matsumoto et al. 1997), reaching a maximum level at the mature green (MG) stage and rapidly decreasing after the breaker stage (Inaba et al. 1980, Rolin et al. 2000, Carrari and Fernie 2006). In a previous study, we reported a role for GABA in climacteric respiration during fruit ripening, and its reduction ratio during ripening was important for GABA levels in fruit (Akihiro et al. 2008, Saito et al. 2008, Yin et al. 2010). However, although GABA-TK activities were much higher than those of GABA-TP in ripening fruits, we could only isolate pyruvate- and glyoxylate-dependent GABA-T (GABA-TP/TG) genes, including *SlGABA-T1*, *SlGABA-T2* and *SlGABA-T3* (Akihiro et al. 2008). Clark et al. (2009b) showed that these proteins exhibited enzymatic activities and localized to the mitochondrion (SlGABA-T1), cytosol (SlGABA-T2) and plastid (SlGABA-T3). These results suggest that GABA-TP/TGs are major isoforms, even in ripening tomato fruits. Therefore, the aim of this study was to determine which GABA-T isoform is responsible for the conversion of GABA to SSA in fruit using RNA interference (RNAi) transgenic plants with suppressed GABA-TP/TG gene expression (*SlGABA-T*^{RNAi}). The results show that *SlGABA-T1* primarily contributes to the GABA conversion in ripening fruit. In addition, systemic GABA-T suppression caused GABA accumulation and affected vegetative and reproductive growth in tomato plants. These findings shed light on the physiological roles of GABA-T in the tomato.

Results

The expression of *SlGABA-T* genes and GABA contents in the leaves and stems of *35S::SlGABA-T*^{RNAi} transgenic lines

First, to evaluate suppression of *SlGABA-T* gene expression through RNAi, quantitative reverse transcription-PCR (qRT-PCR) was performed using leaves (10 weeks after the transplanting) from wild-type (WT) and 35S promoter-driven *SlGABA-T*^{RNAi} (*35S:: SlGABA-T*^{RNAi}) lines (Fig. 2). The expression levels in each line were calculated relative to that of *SlGABA-T1* gene in WT, which was established as 100 %. In WT leaves, the relative expression level of *SlGABA-T2* and *SlGABA-T3* were 2.1 % and 169.4 % compared to *SlGABA-T1* in the WT, respectively (Fig. S3). The expression of *SlGABA-T1* and *SlGABA-T3* genes in *SlGABA-T1*^{RNAi} lines were mostly suppressed compared with that in WT, except for *SlGABA-T1*^{RNAi} line No. 10 (Fig. 2A). The expression levels of the RNAi-targeted genes in the *SlGABA-T1*^{RNAi} lines were 30.6 % (line No. 1), 20.7 % (line No. 2), 5.1 % (line No. 23) and 3.9 % (line No. 28). *SlGABA-T2* expression in the *SlGABA-T1*^{RNAi} was decreased in lines No. 2 and 10; however, expression was increased in lines No. 23 and 28. In the *SlGABA-T2*^{RNAi} and *SlGABA-T3*^{RNAi} lines, the expression levels of *SlGABA-T2* and *SlGABA-T3* genes were suppressed in all of the tested lines (Fig. 2B and C). The relative expression levels of the RNAi-targeted genes were 0.02 % (line No. 21), 0.003 % (line No. 22), 0.01 % (line No. 42), 0.32 % (line No. 48) and 0.52 % (line No. 57) in *SlGABA-T2*^{RNAi} lines and 10.2 % (line No. 2), 0.2 % (line No. 5), 30.2 % (line No. 10), 4.7 % (line No. 20) and 48.9 % (line No. 21) in *SlGABA-T3*^{RNAi} lines. The *SlGABA-T1* expression was decreased in *SlGABA-T2*^{RNAi} lines No. 22 and 42; however, the expression was similar or higher compared with that of the WT plants in other lines. The GABA content was measured in the leaves of the WT and *35S::SlGABA-T*^{RNAi} lines (Fig. 3A). The GABA content in the leaves of the *35S::SlGABA-T*^{RNAi} lines was higher than that in the WT plants. The GABA content was dramatically increased in *35S::SlGABA-T1*^{RNAi} lines No. 1 and 23, which corresponded to a 13.3- to 15.9-fold increase, respectively, over that in WT plants. The GABA content in *35S::SlGABA-T1*^{RNAi} line No. 2 and *35S::SlGABA-T2*^{RNAi} line No. 22 was 7.3 to 7.7 times

higher than that in the WT plants. The GABA content in the *SlGABA-T2*^{RNAi} and *SlGABA-T3*^{RNAi} lines, except for *SlGABA-T2*^{RNAi} line No. 22, was 2.7- to 3.7-fold higher than that in WT. Glutamate content in leaves was also measured in WT and the *35S::SlGABA-T*^{RNAi} lines (Fig. 3B). The leaf glutamate levels were significantly lower in the *35S::SlGABA-T*^{RNAi} lines than that in the WT except for *SlGABA-T1*^{RNAi} line No. 1. The content in *35S::SlGABA-T1*^{RNAi} line No. 2 and *35S::SlGABA-T3*^{RNAi} line No. 10 corresponded to 52.3 % and 52.5 % compared to WT, respectively. In other *35S::SlGABA-T*^{RNAi} lines, it was suppressed to 20.5% to 32.5% compared to the WT.

The GABA and glutamate contents were measured in WT and *35S::SlGABA-T*^{RNAi} lines (Fig. 3A and B). The GABA content in the stems of the *35S::SlGABA-T*^{RNAi} lines was also higher than that in the WT plants. The GABA content in *35S::SlGABA-T1*^{RNAi} lines No. 1, 2 and 23 corresponded to a 10.1-, 8.0- and 11.7-fold increase, respectively, over that in WT plants (Fig. 3A). The GABA content in the *SlGABA-T2*^{RNAi} and *SlGABA-T3*^{RNAi} lines was 1.3 to 3.0 times higher than that in WT. The stem glutamate levels were almost constant in WT and all *35S::SlGABA-T*^{RNAi} lines, except for *35S::SlGABA-T3*^{RNAi} lines No. 5 and 21 (Fig. 3B).

Vegetative growth and flower/fruit setting in the *35S::SlGABA-T*^{RNAi} lines

The vegetative growth of the 10-week-old *35S::SlGABA-T*^{RNAi} lines was also evaluated (Fig.4). As shown in Figs. 4 and 5, transgenic plants with suppressed *SlGABA-T* gene expression showed severe dwarfism and infertility. The plant heights were 59.0 % (No. 1), 41.8 % (No. 2), 61.2 % (No. 10), 68.7 % (No. 23) and 49.3 % (No. 28) suppressed in *SlGABA-T1*^{RNAi} lines and 61.2 % (No. 2), 43.3 % (No. 5), 73.1 % (No. 10), 61.9 % (No. 20) and 71.6 % (No. 21) in *SlGABA-T3*^{RNAi} lines compared with the WT plants (Fig. 4D). However, there was no clear effect on the plant heights in the *SlGABA-T2*^{RNAi} lines, although line No. 42 exhibited a level of dwarfism similar to that of the *SlGABA-T1*^{RNAi} and *SlGABA-T3*^{RNAi} lines.

Although there were no visible morphological changes in the flowers in the transgenic plants, a marked flower abscission was observed in the *SlGABA-T1*^{RNAi} lines (Fig. 5B). The

fruit-setting ratio in total flowering was decreased in most of the *SlGABA-T1*^{RNAi} lines compared with that of the WT, by 27.5 % in line No. 1 and 40.4 % in line No. 28. Line No. 23 exhibited severe infertility and did not set any fruit (Fig. 5E). To confirm infertility in *SlGABA-T1*^{RNAi}, additional tests were performed with *SlGABA-T1*^{RNAi} plants (Fig. S5). The suppression of *SlGABA-T1* gene expression was consistent with the decreased fruit-setting ratio in the additionally tested *SlGABA-T1*^{RNAi} lines (Fig. S5A and B). The fruit-setting ratio was positively correlated with the *SlGABA-T1* mRNA levels in *SlGABA-T1*^{RNAi} plants (Fig. S5C). Flowering in the *SlGABA-T2*^{RNAi} and *SlGABA-T3*^{RNAi} lines was similar to that in the WT (Fig. 5A, C and D). Although the fruit-setting ratio was slightly or moderately decreased in lines No. 22 and 42 in *SlGABA-T2*^{RNAi} and lines No. 5 and 21 in *SlGABA-T3*^{RNAi}, no correlation was observed between the fruit-setting ratio and the expression levels of *SlGABA-T* genes in the both lines (Figs. 2 and 5E).

GABA contents and expression of *SlGABA-T* genes in the fruit of *SlGABA-T*^{RNAi} lines

To determine which isoform is important for GABA metabolism in ripening fruit, the GABA contents and gene expression of *SlGABA-T* genes were analysed in the RNAi lines (Fig. 6-10). The GABA content in *35S::SlGABA-T1*^{RNAi} lines No. 2 and 28 and *35S::SlGABA-T2*^{RNAi} line No. 22 reached 11.5 to 18.1 μmol gFW^{-1} at the MG stage, 12.3 to 19.9 μmol gFW^{-1} at the yellow (Yell) stage and 10.3 to 14.0 μmol gFW^{-1} at the red (Red) stage, which correspond to 1.3- to 2.0-fold higher than the WT in the MG fruits, 2.0- to 3.3-fold higher in the Yell fruits and 6.8- to 9.2-fold higher in the Red fruits, respectively (Fig. 6). In addition, the reduction of the GABA ratio during ripening was changed in the GABA-accumulating lines. When the GABA content at the MG stage was set as 100 %, the ratio was 68.3 % at the Yell stage and 16.9 % at the Red stage in the WT fruits. In contrast, the ratios were 109.9 %, 91.9 % and 107.3 % at the Yell stage and 77.1 %, 74.4 % and 89.8 % at the Red stage in *35S::SlGABA-T1*^{RNAi} lines No. 2 and 28 and *35S::SlGABA-T2*^{RNAi} line No. 22, respectively. However, the GABA contents in the fruits of other RNAi lines were similar or lower than that in the WT. Although the GABA ratios at the Yell stage were different (37.6 –

83.1 %) among these lines, the ratios at the Red stage were 16.4 – 43.1 %, which was reduced compared with that in GABA-accumulating lines. The expression of *SlGABA-T1* was strongly suppressed in the GABA-accumulating lines, at 3.6 % and 4.7 % in *35S::SlGABA-T1*^{RNAi} lines No. 2 and No. 28 and 4.7 % in *35S::SlGABA-T2*^{RNAi} line No. 22 compared with that of the WT (Fig. 7A-C). However, the expression of the *SlGABA-T1* gene in other lines was not changed, and the GABA content was not increased in those lines (Fig. 6 and Fig. 7A-C). The expression of *SlGABA-T2* was significantly suppressed in most of the tested lines except *35S::SlGABA-T2*^{RNAi} line No. 57 and *35S::SlGABA-T3*^{RNAi} line No. 10 (Fig. 7A-C). The expression of *SlGABA-T3* was suppressed in *35S::SlGABA-T1*^{RNAi} line No. 28 and *35S::SlGABA-T3*^{RNAi} lines No. 2 and 5. The expression of RNAi-targeted genes was effectively suppressed in *35S::SlGABA-T2*^{RNAi} line No. 21 and *35S::SlGABA-T3*^{RNAi} lines No. 2 and 5, which corresponds to 14.0 %, 1.6 % and 1.7 % compared with those of the WT, respectively. However, these lines did not show an increase in GABA accumulation (Fig. 6).

To avoid the negative effects of the systemic suppression of *SlGABA-T* genes through the 35S promoter (see Figs. 4 and 5), we also generated *SlGABA-T* RNAi lines through the E8 promoter (*E8::SlGABA-T*^{RNAi}), which is a strong inducible promoter specific to ripening tomato fruit (Deikman et al. 1998). Unlike the *35S::SlGABA-T1*^{RNAi} lines, the *E8::SlGABA-T1*^{RNAi} lines showed a similar phenotype to WT plants and did not show dwarfism or infertility (data not shown). The fruit GABA content at the MG stage in *E8::SlGABA-T1*^{RNAi} lines was 6.9 to 8.5 μmol gFW^{-1}, a similar level to that observed in the WT plants (Fig. 8). However, the fruit GABA content rapidly dropped to approximately 2.2 μmol gFW^{-1} at the Red stage in the WT, *E8::SlGABA-T2*^{RNAi} and *E8::SlGABA-T3*^{RNAi} lines and remained between 4.4 and 5.8 μmol gFW^{-1} in the *E8::SlGABA-T1*^{RNAi} lines, which was approximately 2.5-fold higher than in the WT and other RNAi lines (Fig.8). In WT fruits, the GABA ratio was 50.2 % at the Yell stage and 27.6 % at the Red stage compared with the GABA content at the MG stage. However, the ratios were 97.5 %, 87.3 % and 69.3 % at the Yell stage and 67.2 %, 68.7 % and 64.6 % at the Red stage in *E8::SlGABA-T1*^{RNAi} lines No. 1, 8 and 27,

respectively. In the GABA-accumulating lines, *SlGABA-T1* expression was suppressed (Fig. 9A), and its relative values were 5.1 % (line No.1), 6.1 % (line No.8) and 6.8 % (line No.27) compared with that in the WT plants. The suppression of the *SlGABA-T1* gene was only observed in those lines. Although the expression of *SlGABA-T*2 and *SlGABA-T*3 was suppressed in some lines (No. 1 and 8 of *E8::SlGABA-T1*^{RNAi}, No. 5 and 39 of *E8::SlGABA-T2*^{RNAi} and No. 7, 18 and 57 of *E8::SlGABA-T3*^{RNAi}), there was no correlation between GABA accumulation and gene suppression (Fig. 8, Fig. 9A-C).

Finally, correlations between the GABA contents and the mRNA levels of the *SlGABA-T* genes in *SlGABA-T*^{RNAi} lines were analysed (Fig. 10). The *SlGABA-T1* expression was clearly correlated with the fruit GABA contents in the *35S::SlGABA-T*^{RNAi} lines (Fig. 10A). In the *E8::SlGABA-T*^{RNAi} lines, although the coefficient of determination was lower than that in *35S::SlGABA-T*^{RNAi}, a correlation between *SlGABA-T1* expression and the fruit GABA content was observed. In contrast, there were no correlations between the *SlGABA-T2* and *SlGABA-T3* expression in the *35S::SlGABA-T*^{RNAi} and *E8::SlGABA-T*^{RNAi} lines (Fig. 10B and C).

Amino acid contents in WT and *35S::SlGABA-T*^{RNAi} fruits

The profiles of major and minor amino acids in WT and *35S::SlGABA-T*^{RNAi} fruits were shown in Table 1 and Table S2, respectively. In WT fruit, GABA and glutamine accumulated at MG stage and decreased after breaker stage (Table 1). In contrast, aspartate contents in WT increased after breaker stage. In the GABA-accumulating *35S::SlGABA-T1*^{RNAi} lines such No. 2 and 28 and *35S::SlGABA-T2*^{RNAi} line No. 22 (Fig. 6), the GABA ratio in total amino acids reached 57.7 %, 54.3 % and 36.9 % at the MG stage, respectively (Table 1). Those did not rapidly decrease even after the breaker stage. On the other hand, the aspartate ratio in the GABA-accumulating lines was lower than that in the WT even after the breaker stage. There was a negative correlation between GABA and aspartate. Although GABA is converted to alanine and glycine by GABA-T reaction, alanine and glycine contents did not associate with GABA contents (Table 1).

Whereas total amino acid content in WT decreased at Yell stage compared with that of MG stage, it increased again at Red stage (Table 1). Total amino acid and total protein amino acid levels in MG stage of *35S::SlGABA-T2*^{RNAi} line No. 21 and 57 and, *35S::SlGABA-T3*^{RNAi} line No. 10 were lower than that of WT. GABA contents in these lines were also lower than that in WT. Total amino acids in Red stage were almost constant in WT and all *35S::SlGABA-T*^{RNAi} lines, except for *35S::SlGABA-T3*^{RNAi} lines No. 2 and 10.

Discussion

The suppression of *SlGABA-T* gene expression induced the alteration of phenotypes

To clarify the physiological function of *SlGABA-T* genes, we conducted loss-of-function analyses utilising RNAi transgenic lines with suppressed *SlGABA-T* gene expression. The transgenic plants showed severe abnormal phenotypes, such as dwarfism and infertility. The plant height in the *35S::SlGABA-T1*^{RNAi} and *SlGABA-T3*^{RNAi} lines was half or less than that in the WT plants (Fig. 4A, C and D). On the other hand, no remarkable changes were observed in the *SlGABA-T2*^{RNAi} lines except for line No. 42, in which the expression of *SlGABA-T1* and *SlGABA-T3* were also suppressed (Fig. 2B). Actually, the trigger sequence of each *SlGABA-T*^{RNAi} was designed in the region lying astride between 5'-UTR and ORF. However, the targeting region for *SlGABA-T2*^{RNAi} was mostly included in ORF region and shared a high similarity to other two genes because there is not signal peptide in the N-terminus region (Table S1). This would cause the unexpected suppression on the other isoforms in *35S::SlGABA-T2*^{RNAi} lines.

The abnormal phenotypes have also been reported in transgenic tobacco lines overexpressing the C-terminal-truncated GAD gene, which over-accumulated GABA (Baum et al. 1996, Akama and Takaiwa 2007). In those transgenic plants, a decrease of glutamate associated with the GABA increase was observed. In this study, although the similar tendency was observed in

leaves of *35S::SlGABA-T*^{RNAi} lines in the glutamate and GABA contents, there was no quantitative correlation to the dwarfism (Figs. 3 and 4). In addition, whereas the GABA content was increased, the glutamate content was almost constant in stem of *35S::SlGABA-T*^{RNAi} lines compared with that in WT (Fig. 3). Renault et al. (2011) reported that excessive GABA accumulation negatively affected cell elongation in the hypocotyl through the down-regulation of cell-wall-related gene expression, such as the genes encoding arabinogalactan, expansin and tonoplast intrinsic proteins. The vertical cell size of stem cortex tissue in the *35S::SlGABA-T1*^{RNAi} and *35S::SlGABA-T3*^{RNAi} lines was obviously smaller than that of WT whereas it was almost similar in the *35S::SlGABA-T2*^{RNAi} line (Fig. S4). This tendency was consistent with the results in plant heights (Fig. 4), indicating the dwarf phenotype observed in the present research also result from defects in cell elongation and SlGABA-T2 would not be involved in this event. However, the plant heights were similar between *35S::SlGABA-T1*^{RNAi} and *35S::SlGABA-T3*^{RNAi} individuals, although the GABA contents in *35S::SlGABA-T3*^{RNAi} leaves were much lower than that in *35S::SlGABA-T1*^{RNAi} plants (Fig. 3 and Fig. 4D), suggesting that other factors are involved in the dwarf phenotype.

A severe abscission of flowers was observed in the *35S::SlGABA-T1*^{RNAi} lines (Fig. 5B). Therefore, we compared the fruit-setting ratios between the WT and RNAi transgenic plants (Fig. 5). The fruit-setting ratios in *35S::SlGABA-T1*^{RNAi} lines No. 1, 23 and 28 were markedly decreased compared with that of the WT (Fig. 5E). The lower fruit-setting ratio and positive correlation with the *SlGABA-T1* mRNA levels were also confirmed in additionally tested *SlGABA-T1*^{RNAi} plants (Fig. S5). Indeed, the subcellular localization was previously shown to be different for each SlGABA-T protein, with SlGABA-T1 localized in the mitochondria (Clark et al. 2009b). However, these phenomena were not observed in *E8::SlGABA-T1*^{RNAi} plants in the present study (data not shown). The *E8* promoter is a fruit-ripening-specific promoter in the tomato, and it does not control gene expression in flowers (He et al. 2008). The inhibition of pollen tube growth and a reduction of seed fertility have been reported in a *GABA-TP/TG* knockdown mutant/transgenic *Arabidopsis* plant, which also showed that GABA-TP/TG is localized in the mitochondria (Palanivelu et al.

2003, Mirabella et al. 2007, Renault et al. 2011, Clark et al. 2009a). These results indicate that impair of mitochondrial localized GABA-T1 would cause aberrant GABA accumulation in cytosol and result in aberrant plant development.

Change of amino acid contents in tomato fruits in *SlGABA-T*^{RNAi} lines

GABA is most abundant amino acid in tomato fruits at MG stage, and the content associates with total amino acids by Yell stage (Rolin et al. 2000, Akihiro et al. 2008). In the present study, in the GABA-accumulating *35S::SlGABA-T1*^{RNAi} lines No. 2 and 28 and *35S::SlGABA-T2*^{RNAi} line No. 22 (Fig. 6), the GABA ratio in total amino acids reached 57.7 %, 54.3 % and 36.9 %, respectively, at the MG stage (Table 1), and the GABA content did not rapidly decrease, even after the breaker stage. However, the aspartate ratio in the GABA-accumulating lines was lower than that in the WT after the breaker stage (Table 1). Accumulations of glutamate and asparatate after breaker stage in tomato fruits have been reported in previous studies (Rolin et al. 2000, Roessner-Tunali et al. 2003, Mattoo et al. 2006, Mounet et al. 2007). In this study, there was a negative correlation between the accumulation of GABA and aspartate at Red stage (Table 1). However, total amino acids accumulated in the Red stage were almost identical between the WT and the GABA-accumulating lines. The reduced aspartate content has been reported in the GABA-rich tomato cultivar ‘DG03-9’ (Saito et al. 2008, Akihiro et al. 2008). These results suggest that both GABA and aspartate are synthesised from glutamate, and the accumulation of GABA after the ripening stage prevents aspartate accumulation.

GABA was converted to alanine and glycine by GABA-T reaction (Clark et al., 2009a; Clark et al., 2009b). In this study, alanine and glycine contents in the GABA-accumulating lines were not changed compared with that of WT (Table 1). Because the absolute values of these amino acids were lower in the fruits of WT, those would be rapidly converted to other amino acids in tomato fruit. However, at the Yell stage, all of GABA, glutamine and total amino acid levels in WT decreased from MG stage. Our previous work showed GABA is converted to organic acids during

ripening (Yin et al. 2010). However, it has not been fully understood what are those amino acids converted to during Yell stage. It would be interesting to perform metabolome analyses focusing on the primary metabolites utelizing the *SlGABA-T*^{RNAi} lines.

The isoform responsible for GABA conversion in tomato plants

It has been accepted that GABA is catabolised in the mitochondria (Bouchè and Fromm 2004). However, Clark et al. (2009b) reported three GABA-T1-3 enzymes in tomato that were localized in the mitochondrion, cytosol and plastid, with each isoform predicted to have unique functions.

In the present study, the leaf GABA contents in all *35S::SlGABA-T*^{RNAi} lines were higher than that in the WT plants (Fig. 3). The *SlGABA-T1* expression was decreased in *SlGABA-T1*^{RNAi} plants and *SlGABA-T2*^{RNAi} lines No. 22 and 42 but not in *SlGABA-T1*^{RNAi} line No. 10 (Fig. 2A-C). However, *SlGABA-T1* expression was similar or higher compared with that of the WT plants in other lines. In those lines, the expression of *SlGABA-T2* and *SlGABA-T3* genes was suppressed (Fig. 2B and C). These results suggest that *SlGABA-T2* or *SlGABA-T3* is involved in GABA metabolism in tomato leaves. In tomato fruits, the GABA contents in *35S::SlGABA-T1*^{RNAi} lines No. 2 and 28 and *SlGABA-T2*^{RNAi} line No. 22 were 1.3 to 2.0 times higher in MG fruit and 6.8 to 9.2 times higher in Red fruit, respectively, compared with that of the WT (Fig.6). In these lines, the expression of *SlGABA-T1* and *SlGABA-T2* genes was suppressed (Fig. 7A, B). However, excessive GABA accumulation was not observed in other *SlGABA-T2* and *SlGABA-T3* suppression lines (Fig. 6). A clear correlation between fruit GABA contents and *SlGABA-T1* expression level was observed (Fig. 10A), whereas there was almost no correlation with *SlGABA-T2* and *SlGABA- T3* genes (Fig. 10B and C). The same results were obtained through the analyses of *E8*-promoter-driven transgenic lines (Figs. 8, 9 and 10). Indeed, the enzymatic activity of SlGABA-T1 is highest among the three isoforms in the tomato (Clark et al. 2009b). These results clearly indicate that *SlGABA-T1* is

primarily responsible for GABA metabolism in tomato fruits. Unexpectedly, GABA-TP/TG activities in fruit were not significantly different between the *35S::SlGABA-T1*^{RNAi} lines and WT (Fig. S6B) although fruit GABA contents in the RNAi lines clearly increased (Fig. S6A). Because the enzyme assay was performed utilizing crude protein extracted from fruits, the GABA-TP/TG activity corresponding to each isoform could not be separately evaluated. Considering the increase of *SlGABA-T3* expression level in the *35S::SlGABA-T1*^{RNAi} lines No. 2 (Fig. 7), those results would be the outcome of masking by other isoforms.

A previous study demonstrated that GABA-TP/TG was present in the cytosol and plastids (Clark et al. 2009b). Although the physiological functions of these genes are unclear, our results show that these genes function *in vivo* (Figs. 2 and 3). For example, the *35S::SlGABA-T3*^{RNAi} lines showed severe dwarfism (Fig.4). GABA reduction through *SlGABA-T2* and *SlGABA-T3* was observed in the tomato leaves (Figs. 2 and 3), but it was not observed in the fruits (Figs. 6, 7 and 10). The expression of *SlGABA-T1* was correlated with the fruit-setting ratio and GABA accumulation in tomato fruits (Figs. 10A and S5). Thus, *SlGABA-T1* is likely the predominant isoform in tomato flowers and fruits. These results suggest that the three GABA-T genes cooperatively function during the vegetative phase, and GABA reduction occurs through *SlGABA-T1* in the reproductive phase.

In previous work, we reported that *SlGABA-T1* expressed in fruit at all developmental stage whereas those of *SlGABA-T2* and *SlGABA-T3* decreased in Red stage (Akihiro et al. 2008). However, Clark et al. (2009b) reported that the expression levels of all three *GABA-T* genes were low at MG stage, then only *SlGABA-T1* expression significantly increased after breaker stage. In the present study, the accumulation of GABA in *35S::SlGABA-T1*^{RNAi} lines were observed at not only Yell and Red stages but MG stage (Fig. 6). The reduction ratio of GABA in those lines during ripening was also lower than that in WT although SlGADs has already down-regulated after the breaker stage (Akihiro et al. 2008). Those results suggest that the conversion of GABA by SlGABA-T1 has proceeded at least MG stage and increases during ripening. The expression pattern of *SlGABA-T2* and *SlGABA-T3* were not correlated with GABA accumulation during fruit

developmental stages (Akihiro et al. 2008). In the present study, from the point of view of transcription levels, an essential role of SlGABA-T1, but not SlGABA-T2 and SlGABA-T3, in fruit GABA level was demonstrated through the loss of function analyses (Fig. 6, 7 and 10). On the other hand, the expression level of *SlGABA-T3* was higher than that of *SlGABA-T1* in WT leaves (Fig. S3). It is likely that SlGABA-T3 is involved in the regulation of GABA level in leaf and stem tissues as well as SlGABA-T1.

GABA-TP/TG plays an important role for GABA metabolism in tomato fruits

In our previous work, we reported a negative correlation between GABA contents and GABA-TK activity in tomato fruits through a comparison between ordinary and GABA-rich cultivars (Akihiro et al. 2008). A recent study suggested that decreased GABA-TK activity causes GABA accumulation in tomatoes stored under low-O_2 conditions (Mae et al. 2012). However, Clark et al. (2009b) reported that all three *SlGABA-T*-encoded proteins showed only GABA-TP/TG activities and pointed out a possibility that the above researches had detected artificial GABA-TK activity. Deewatthanawong et al. (2010) suggested that higher GABA concentrations in CO_2-treated fruits were due to a decreased GABA-TP activity. In the present study, we demonstrated *SlGABA-T1* is important for GABA metabolism in the tomato fruit. Although we previously reported the importance of GABA-TK in the tomato fruit, the gene encoding this protein has not yet been identified in the tomato. Therefore, based on the loss-of-function experiments performed in the present study, we now conclude that GABA-TP/TG is an essential factor for GABA metabolism in tomato plants.

In contrast to *SlGABA-T1*, the physiological functions of *SlGABA-T2* and *T3* remain unclear. In the tomato and other species, a possibility of alternative pathway for the breakdown of SSA *via* gamma-hydroxy butyric acid (GHB) production has been reported (Clark et al. 2009b). Although GABA-derived SSA is primarily reduced through SSADH activity in the mitochondria,

SSA is also converted to GHB through the activity of succinate semialdehyde reductase (SSR) (Bouché and Fromm 2004). In *Arabidopsis*, glyoxylate reductase, which is identical with SSR (Shelp et al. 2012), is localized in the cytosol and plastids (Hoover et al. 2007, Simpson et al. 2008). Two *SlSSR* genes have been isolated in the tomato (Akihiro et al. 2008); however, the localization of these genes has not been analysed. If SlSSR1 and -2 are localized to the cytosol and plastids, *SlGABA-T2* and *-T3* participate in an additional route for SSA metabolism (Fig. 1). However, further characterisation of the RNAi transgenic plants will be required to clarify SSA metabolism.

In the present study, we successfully generated GABA-over-accumulating tomato plants through the suppression of GABA-T genes and demonstrated that *SlGABA-T1* is the most essential isoform for GABA metabolism in tomato fruits. The results of this study will be available for screening GABA-rich mutants, which will be an excellent bioresource for breeding a new GABA-rich tomato cultivar.

Materials and methods

Plant materials and growth conditions

The tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom was used in this study. Germinated seedlings were transplanted into rockwool and grown in a culture room at 25 ˚C under 16 h light / 8 h dark conditions. For *SlGABA-T*^{RNAi} lines, the shoots derived from calli were transplanted. The plants were fed a standard nutrient solution (Otsuka House. No. 1 and 2, Otsuka Chemical CO, Japan). The plant height measurements and leaf samples were obtained at 10 weeks after transplantation. The fruit-setting ratio was calculated from the number of total fruits set and total flowering. The fruits were sampled at 24 to 27 days after flowering (DAF), 28 to 33 DAF and 42 to 45 DAF to obtain three development stages: MG, Yell and Red, respectively. In this study, only the T_0 generation plants were analysed because *SlGABA-T* suppression caused severe infertility in

transgenic plants.

Vector construction and transformation

The RNAi constructs used to suppress the mRNA expression of each *SlGABA-T* gene were created under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter or the fruit-specific *E8* promoter (Fig. S1). To create RNAi constructs targeted towards *SlGABA-T1* suppression, the RNAi-targeted region of *SlGABA-T1* was amplified using gene-specific primers (shown in Table S1). The RNAi-targeted region of about 300 bp was designed at 5' side of *SlGABA-T* gene, this one was contained untranslated region and open reading frame (Fig. S2). The PCR fragment was directly cloned into the entry vector pCR8/GW/TOPO (Invitrogen) and transferred into the Gateway vector pBI sense-antisense GW (Inplanta Innovations) using the Gateway LR Clonase enzyme (Invitrogen). This construct was designated *SlGABA-T1*^{RNAi}. The same strategy was used to create RNAi constructs for the suppression of other *SlGABA-T* genes using specific primers (shown in Table S1). To create RNAi constructs under the control of the *E8* promoter, this region (accession number AF515784) was amplified using specific primers containing *Bln* I and *Xho* I sites. The fragment was cloned in place of the CaMV 35S promoter in the pBI sense-antisense GW vector. Subsequent procedures were performed using this same strategy. These constructs were then transformed into *Agrobacterium tumefaciens* GV2260 using the electroporation method. The constructs were transformed into WT 'Micro-Tom' using the agrobacterium method (Sun et al. 2006). The transgenic plants were selected on Murashige and Skoog (MS) agar plates containing kanamycin (100 mg l^{-1}).

Extraction and measurement of GABA and amino acid contents

Approximately 50 mg of fresh sample was homogenised in liquid nitrogen using a mortar and pestle, and subsequently, 500 µl of 8 % (w/v) trichloroacetic acid was added. The samples were centrifuged at 10,000 ×*g* for 20 min at 4 ˚C. The supernatant was transferred into a fresh tube, 400 µl

of pure diethyl ether was added and the tube was mixed vigorously for 10 min. The samples were centrifuged again at 10,000 ×*g* for 10 min at 4 ˚C. The supernatant was removed, and 400 µl of diethyl ether was added. The samples were mixed vigorously for 10 min and centrifuged at 10,000 ×*g* for 10 min at 4 ˚C. The supernatant from this centrifuge step was removed and incubated under a draft of air for 30 min for the complete evaporation of diethyl ether. The samples for amino acid analysis were evaporated using an evaporator (CVE3100, TOKYO RIKAKIKAI), and 300 µl of water was added. This procedure was repeated twice. The samples were dissolved in 0.1 N HCl for the amino acid analysis (JLC-500/V2, Japan Electron Optics Laboratory). The 'GABase' assay for GABA was performed using the method described by Jakoby (1962) with slight modifications. In the 'GABase' assay, the reduction of NADP to NADPH was monitored spectrophotometrically at 340 nm, pH 8.6 at 37 ˚C, as a function of time using GABA as a substrate.

Quantitative expression analysis

Total RNAs were extracted from tomato plants using the RNeasy Plant Mini kit (Qiagen) and digested using DNase I (NipponGene) according to the manufacturer's instructions. Approximately 1 µg of total RNA was used to synthesise single-strand cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen). The mRNA expression of each *SlGABA-T* gene was analysed using qRT-PCR. The qRT-PCR experiments were performed using a Takara Thermal Cycler Dice Real-Time System with SYBR Premix Ex Taq II (Takara). The qRT-PCR was performed with gene-specific primers (supplementary Table S1). For the PCR amplification, the cDNA was denatured at 94 °C for 30 sec in the first cycle, followed by 45 cycles of denaturing for 5 sec, primer annealing at 55 °C for 10 sec and extension at 72 °C for 15 sec. The mRNA levels of each *SlGABA-T* were determined relative to the control Ubiquitin (UBQ) (accession number X58253) mRNA according to the methods of Kim et al. (2010).

Histological analysis

The plants grown from cuttings of T_0 generation were used for histological analysis. Longitudinal sections of stem tissue were obtained by hand-cutting with razor blade. The sections were immediately stained with 0.1 % toluidine blue for 15 min and then rinsed with distilled water. The samples were mounted on a slide glass and observed by optical microscope (BX53, OLYMPUS).

GABA-T enzymatic assay

GABA-T enzymatic assay was performed according to the procedure described by Clark et al. (2009a, 2009b). Five g of fresh tomato fruit obtained from T_0 *35S::SlGABA-T1*^{RNAi} plants were homogenized with a mortar and pestle in a five-fold volume of ice-cold extraction buffer [50 mM Tris-HCl (pH 8.2), 3 mM dithiothreitol, 1.25 mM EDTA, 2.5 mM $MgCl_2$, 10 % (v/v) glycerol, 6 mM 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonate, 1mM phenylmethylsulphonylfluoride, 2.5 μg ml^{-1} of leupeptin and pepstatin A, 2 % (w/v) polyvinylpyrrolidone and 2 μg ml^{-1} of pyridoxial-5-phosphate]. The homogenates were centrifuged at 10,000 x g for 15 min at 4 °C, and the pellet was discarded. The supernatant was concentrated using Amicon ultra-4 (10kDa, Millipore).The extract was desalted using PD-10 columns (GE healthcare) that were equilibrated in the extraction buffer before an use. GABA-TP and GABA-TG activity were measured as GABA-dependent alanine and glycine production, respectively. For the assay, 100 μl of the crude protein was used in total 500 □μl reaction mixture [50 mM N-tris(hydroxymethyl)methyl-4-aminobutanesulphonic acid (TABS, pH 9.0), 1.5 mM dithiothreitol, 0.625 mM EDTA, 0.1 mM pyridoxial-5-phosphate, 10 % (v/v) glycerol, 1 mM GABA and 1 mM pyruvate or glyoxylate]. The reaction solution was incubated at 30 °C for 6 hours and then terminated by the addition of ice-cold sulphosalicylic acid to a final concentration of 60 mM (Van Cauwenbergue and Shelp 1999). The supernatant was neutralized with NaOH and the resultant alanine and glycine were measured by the HPLC amino acid analyzer (JLC-500/V2, JEOL).

Funding

This work was supported through funding from a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Fellows (No. 225336); through funding from the “JSPS Bilateral Joint Research Project” and the “Japan-France Joint Laboratory Project”, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; and through the “Research and Development Program for New Bio-industry Initiatives (BRAIN)”.

Acknowledgements

The authors would like to thank all laboratory members for their help, advice and discussions. ‘Micro-Tom’ seeds (accession number TOMJPF00001) were obtained from the Gene Research Center, University of Tsukuba, through the National Bio-Resource Project (NBRP) of MEXT, Japan.

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Table 1. Amino acid contents (μmol gFW^{-1}) in WT and *35S::SlGABA-T*^{RNAi} fruits

			35S::SlGABA-T1^{RNAi}			*35S::SlGABA-T2*^{RNAi}			*35S::SlGABA-T3*^{RNAi}		
		WT	2	10	28	21	22	57	2	5	10
MG	Asp	1.01± 0.05	1.26± 0.12	1.09± 0.04	0.93± 0.05	0.40± 0.03	1.55± 0.10	0.58± 0.08	0.88± 0.02	1.12± 0.04	0.70± 0.04
	Asn	3.03± 0.18	2.12± 0.20	2.71± 0.17	1.74± 0.11	0.74± 0.03	4.72± 0.21	1.29± 0.17	3.44± 0.15	5.83± 0.20	2.32± 0.10
	Glu	1.20± 0.12	1.10± 0.11	1.06± 0.04	1.05± 0.06	0.63± 0.05	1.76± 0.06	0.78± 0.12	1.26± 0.06	1.39± 0.07	0.96± 0.04
	Gln	5.49± 0.27	5.22± 0.65	3.61± 0.21	4.06± 0.34	1.51± 0.07	12.63± 0.85	3.29± 0.38	7.50± 0.35	8.67± 0.35	3.89± 0.21
	Gly	0.14± 0.01	0.14± 0.00	0.08± 0.01	0.13± 0.01	0.05± 0.00	0.18± 0.01	0.06± 0.01	0.11± 0.00	0.14± 0.00	0.08± 0.01
	Ala	0.28± 0.03	0.22± 0.02	0.29± 0.03	0.22± 0.01	0.16± 0.01	0.29± 0.01	0.17± 0.02	0.29± 0.00	0.30± 0.01	0.21± 0.01
	GABA	6.77± 0.13	17.29± 1.55	6.17± 0.28	12.52± 0.55	5.34± 0.38	14.95± 0.75	2.91± 0.36	5.98± 0.04	6.05± 0.20	4.18± 0.28
	total	**20.13± 0.84**	**29.95± 2.86**	**16.83± 0.81**	**23.03± 1.25**	**9.64± 0.59**	**40.48± 2.17**	**10.23± 1.30**	**21.25± 0.61**	**26.68± 0.95**	**13.86± 0.69**
	total (-GABA)	**13.36± 0.71**	**12.66± 1.31**	**10.65± 0.53**	**10.52± 0.70**	**4.30± 0.22**	**25.53± 1.42**	**7.32± 0.94**	**15.27± 0.57**	**20.63± 0.75**	**9.68± 0.41**
Yell	Asp	5.29± 0.11	1.65± 0.12	5.19± 0.23	3.83± 0.21	1.55± 0.02	1.56± 0.10	7.02± 0.12	3.35± 0.13	3.53± 0.17	1.14± 0.03
	Asn	1.22± 0.08	2.34± 0.12	1.78± 0.05	1.60± 0.09	0.79± 0.01	1.23± 0.11	1.85± 0.04	2.50± 0.11	1.67± 0.07	2.79± 0.08
	Glu	0.75± 0.03	0.88± 0.04	0.81± 0.00	0.66± 0.02	0.56± 0.02	0.63± 0.05	1.12± 0.01	0.96± 0.06	0.86± 0.05	0.96± 0.03
	Gln	3.04± 0.21	3.73± 0.18	2.58± 0.10	2.84± 0.18	1.37± 0.14	3.05± 0.22	4.43± 0.11	5.81± 0.33	3.50± 0.15	4.50± 0.53
	Gly	0.06± 0.01	0.08± 0.00	0.05± 0.00	0.06± 0.00	0.04± 0.00	0.05± 0.00	0.05± 0.01	0.07± 0.00	0.04± 0.01	0.07± 0.00
	Ala	0.20± 0.00	0.20± 0.01	0.14± 0.01	0.10± 0.00	0.16± 0.00	0.17± 0.01	0.13± 0.01	0.20± 0.01	0.13± 0.01	0.20± 0.00
	GABA	4.58± 0.20	20.91± 0.85	3.59± 0.21	12.94± 0.46	4.20± 0.07	14.77± 0.92	1.91± 0.01	1.91± 0.06	2.72± 0.10	3.00± 0.09
	total	**16.01± 0.51**	**31.60± 1.09**	**15.12± 0.49**	**23.06± 0.92**	**9.35± 0.11**	**22.28± 1.43**	**17.57± 0.12**	**15.91± 0.72**	**13.30± 0.60**	**13.89± 0.78**
	total (-GABA)	**11.43± 0.31**	**10.69± 0.23**	**11.54± 0.28**	**10.12± 0.47**	**5.15± 0.04**	**7.51± 0.51**	**15.66± 0.11**	**13.99± 0.66**	**10.58± 0.50**	**10.88± 0.70**
Red	Asp	15.82± 0.80	7.26± 0.45	16.15± 0.78	8.89± 0.18	12.41± 0.95	7.81± 1.03	14.10± 0.22	24.13± 0.89	14.33± 1.05	7.92± 0.65
	Asn	0.84± 0.04	1.35± 0.11	1.72± 0.08	1.40± 0.03	1.69± 0.13	1.37± 0.20	1.92± 0.03	4.55± 0.18	2.67± 0.18	1.62± 0.13
	Glu	1.83± 0.01	1.26± 0.04	1.88± 0.07	1.32± 0.03	1.64± 0.09	1.27± 0.18	1.86± 0.02	2.69± 0.07	1.97± 0.13	1.30± 0.10
	Gln	1.54± 0.10	2.46± 0.16	2.08± 0.09	2.65± 0.02	3.36± 0.25	3.08± 0.42	4.56± 0.06	8.17± 0.20	3.93± 0.30	2.15± 0.07
	Gly	0.04± 0.00	0.07± 0.00	0.06± 0.00	0.11± 0.00	0.09± 0.00	0.07± 0.01	0.06± 0.01	0.10± 0.01	0.06± 0.01	0.07± 0.01
	Ala	0.27± 0.01	0.36± 0.01	0.25± 0.00	0.47± 0.04	0.42± 0.03	0.38± 0.05	0.30± 0.01	0.44± 0.02	0.29± 0.02	0.31± 0.03
	GABA	1.16± 0.22	13.59± 0.36	1.21± 0.04	11.12± 0.18	1.57± 0.11	9.94± 1.30	1.22± 0.06	0.98± 0.03	1.73± 0.16	0.80± 0.14
	total	**22.72± 0.77**	**27.57± 1.14**	**24.62± 1.12**	**27.44± 0.31**	**22.85± 1.67**	**25.13± 3.32**	**25.30± 0.17**	**43.52± 1.47**	**26.57± 1.95**	**15.19± 1.03**
	total (-GABA)	**21.56± 0.55**	**13.97± 0.79**	**23.41± 1.09**	**16.32± 0.13**	**21.28± 1.55**	**15.19± 2.01**	**24.07± 0.11**	**42.55± 1.44**	**24.83± 1.79**	**14.39± 0.89**

The labels above the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3).

Figure legends

Fig. 1 GABA shunt metabolic pathway

GDH: glutamate dehydrogenase; GAD: glutamate decarboxylase; GABA-TK: α-ketoglutarate-dependent GABA transaminase; GABA-T1, 2 and 3: pyruvate- and glyoxylate-dependent GABA transaminase; SSADH: succinic semialdehyde dehydrogenase; SSR: succinic semialdehyde reductase. The pathways presented by dotted lines indicate predicted pathways based on the localization of each enzyme reported in *Arabidopsis* (Hoover et al. 2007, Simpson et al. 2008).

Fig. 2 Expression levels of *SlGABA-T* genes in *SlGABA-T*^{RNAi} transgenic leaves

(A) *SlGABA-T1*^{RNAi} (open column), (B) *SlGABA-T2*^{RNAi} (shaded column) and (C) *SlGABA-T3*^{RNAi} (closed column). The expression of *SlGABA-T1*, *SlGABA-T2* and *SlGABA-T3* is shown from the top. The labels below the horizontal axis indicate genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT was determined using Student's *t*-test (**P*<0.05, ***P*<0.01).

Fig. 3 GABA and glutamate contents in leaves and stems in WT and *35S::SlGABA-T*^{RNAi} lines

(A) GABA and (B) glutamate contents. The open and closed columns indicate the contents in leaves and stems, respectively. The labels below the horizontal axis indicate genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with WT was determined using Student's *t*-test (**P*<0.05, ***P*<0.01).

Fig. 4 Vegetative growth of 10-week-old *35S::SlGABA-T*^{RNAi} plants

(A) *SlGABA-T1*^{RNAi}, (B) *SlGABA-T2*^{RNAi} and (C) *SlGABA-T3*^{RNAi}. The plants on the far left in each panel are WT. The bars = 5 cm. (D) The plant height of 10-week-old WT and RNAi transgenic lines.

The labels below the horizontal axis indicate the genotypes of the transgenic lines.

Fig. 5 Flowering and fruit setting in *35S::SlGABA-T*^{RNAi} lines

(A) WT, (B) *SlGABA-T1*^{RNAi}, (C) *SlGABA-T2*^{RNAi} and (D) *SlGABA-T3*^{RNAi}. Scale bars = 1 cm. (E) The fruit-setting ratio in the WT and *35S::SlGABA-T*^{RNAi} lines. The labels below the horizontal axis indicate the genotypes of the transgenic lines.

Fig. 6 The GABA contents in the fruits of *35S::SlGABA-T*^{RNAi} lines

The open, shaded and closed columns indicate the MG, Yell and Red stages, respectively. The labels below the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT at each stage was determined using Student's *t*-test (*P<0.05, **P<0.01).

Fig. 7 Expression levels of *SlGABA-T* genes in fruits of *35S::SlGABA-T*^{RNAi} lines

(A) *SlGABA-T1*^{RNAi} (open column), (B) *SlGABA-T2*^{RNAi} (shaded column) and (C) *SlGABA-T3*^{RNAi} (closed column). The expression of *SlGABA-T1*, *SlGABA-T2* and *SlGABA-T3* is shown from the top. The labels below the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT was determined using Student's *t*-test (*P<0.05, **P<0.01).

Fig. 8 The GABA contents in the fruits of *E8::SlGABA-T*^{RNAi} lines

The open, shaded and closed columns indicate the MG, Yell and Red stages, respectively. The labels below the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT at each stage was determined using Student's *t*-test (*P<0.05, **P<0.01).

Fig. 9 Expression levels of *SlGABA-T* genes in fruits of *E8::SlGABA-T*^{RNAi} lines

(A) *SlGABA-T1*^{RNAi} (open column), (B) *SlGABA-T2*^{RNAi} (shaded column) and (C) *SlGABA-T3*^{RNAi} (closed column). The expression of *SlGABA-T1*, *SlGABA-T2* and *SlGABA-T3* is shown from the top. The labels below the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT was determined using Student's *t*-test (**P*<0.05, ***P*<0.01).

Fig. 10 Correlation between fruit GABA contents at the Red stage and *SlGABA-T* mRNA levels in *SlGABA-T*^{RNAi} lines

(A) *SlGABA-T1*, (B) *SlGABA-T2* and (C) *SlGABA-T3*. The open, closed and shaded rhombuses indicate WT, *35S::SlGABA-T*^{RNAi} and *E8::SlGABA-T*^{RNAi} lines, respectively.

Fig. 1

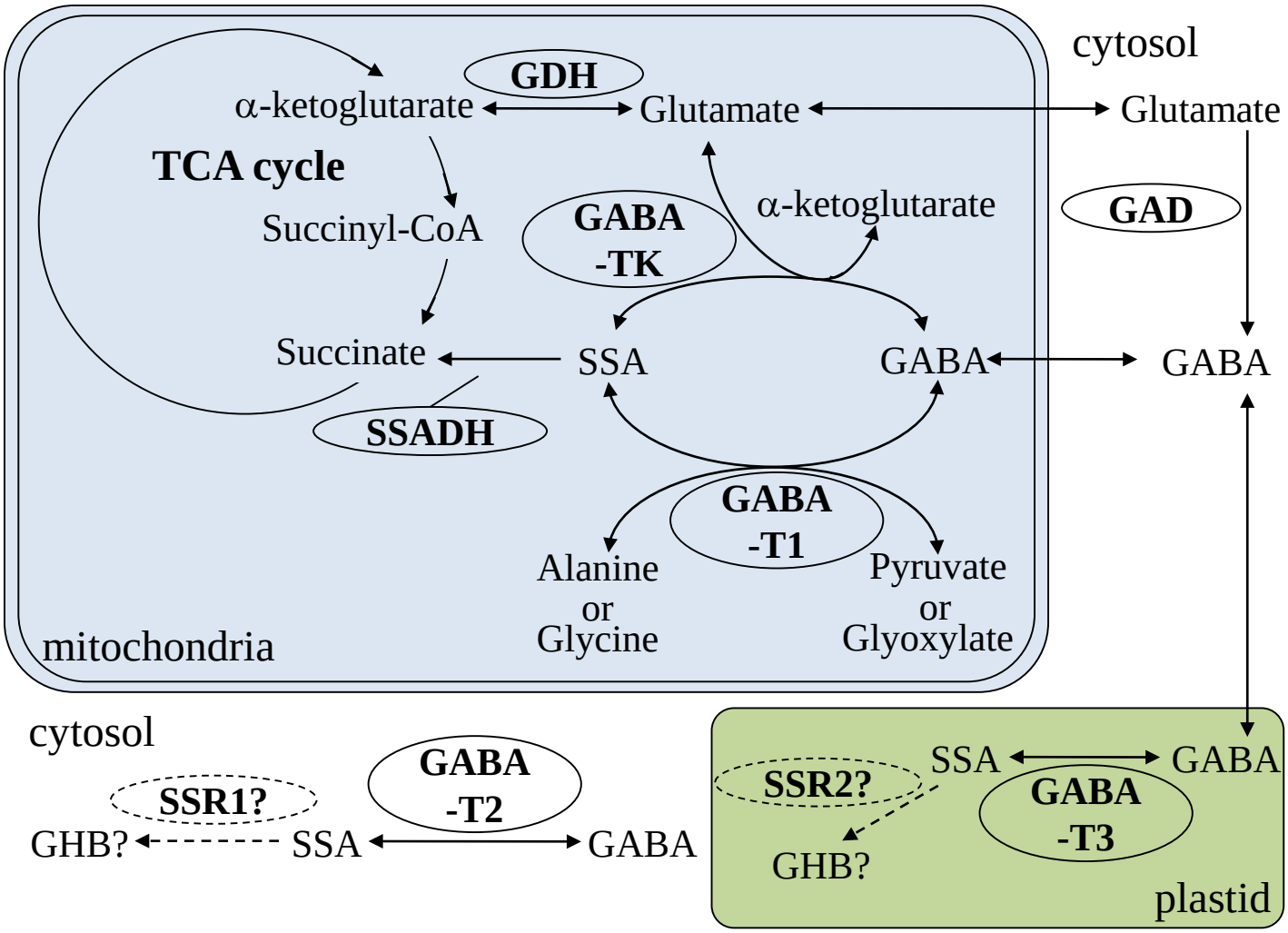

cytosol
GDH
α-ketoglutarate
Glutamate
Glutamate
TCA cycle
Succinyl-CoA
GABA
-TK
α-ketoglutarate
GAD
Succinate
SSA
GABA
GABA
SSADH
GABA
-T1
Alanine
or
Glycine
Pyruvate
or
Glyoxylate
mitochondria
cytosol
SSR1?
GABA
-T2
GHB?
SSA
GABA
SSA
GABA
SSR2?
GABA
-T3
GHB?
plastid

Fig. 2

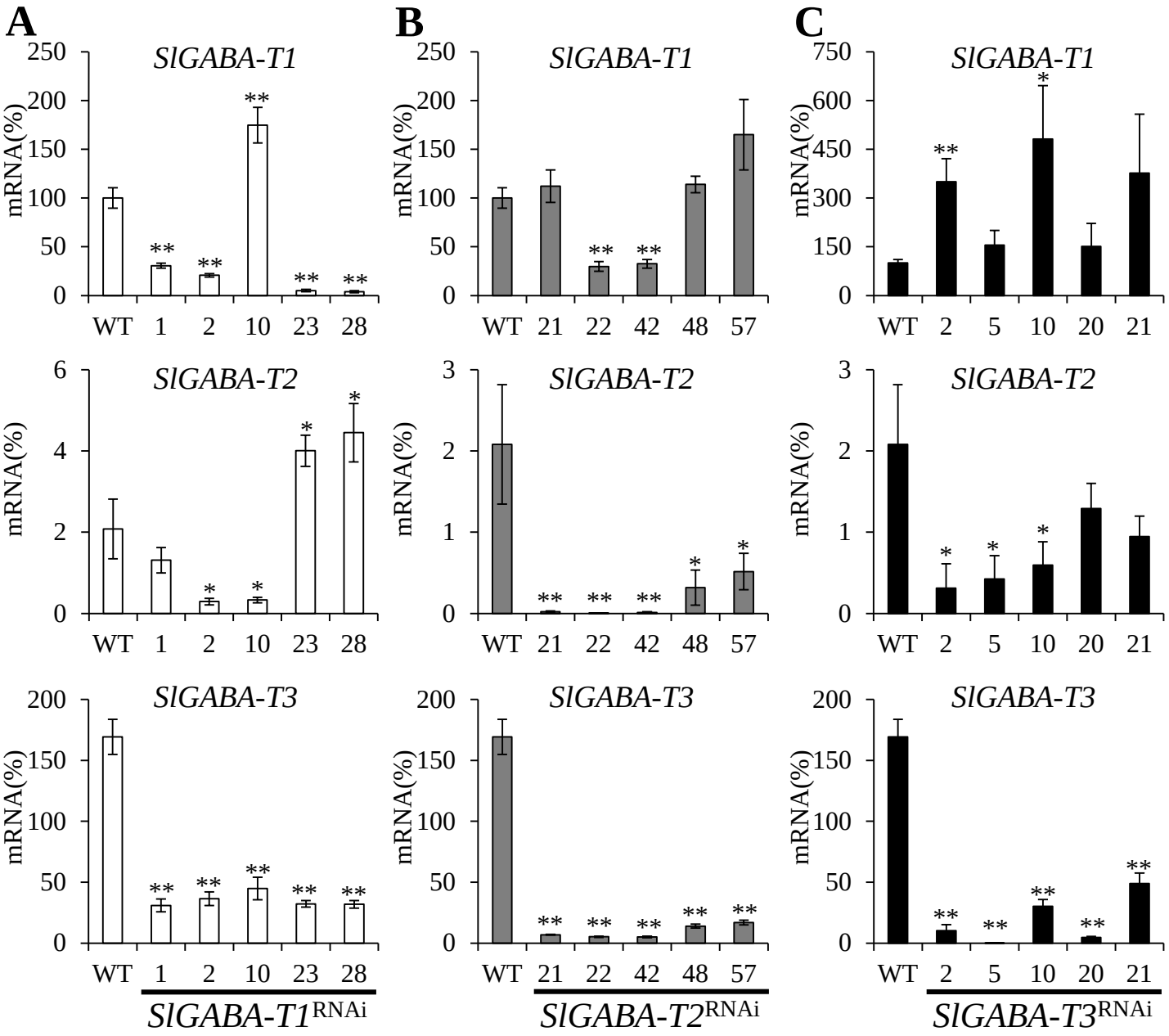

A
B
C
SlGABA-T1
SlGABA-T2
SlGABA-T3
mRNA(%)
WT
1
2
10
23
28
21
22
42
48
57
5
20
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig. 3

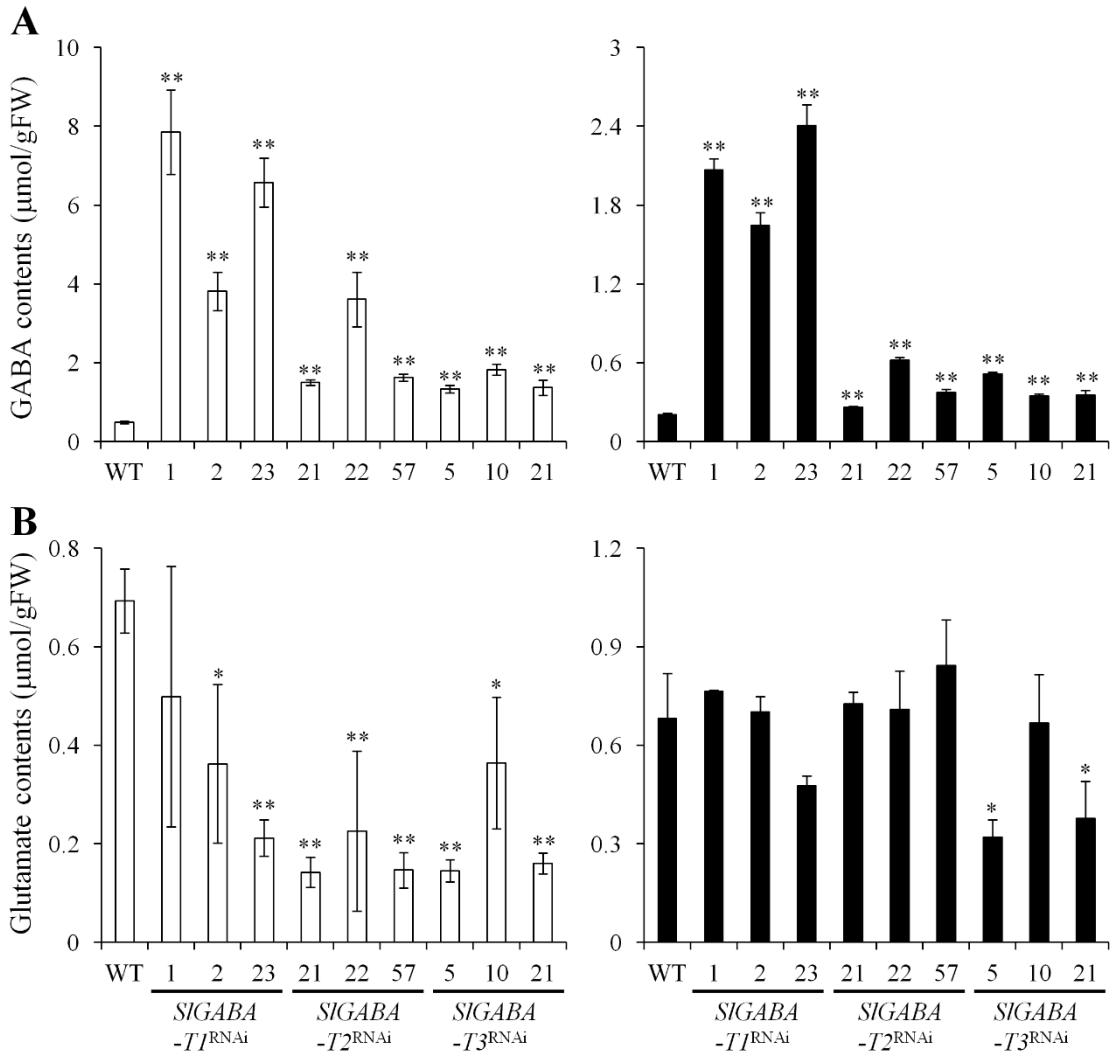

A
GABA contents (μmol/gFW)
10
8
6
4
2
0
3
2.4
1.8
1.2
0.6
0
WT 1 2 23 21 22 57 5 10 21
B
Glutamate contents (μmol/gFW)
0.8
0.6
0.4
0.2
0
1.2
0.9
0.6
0.3
0
WT 1 2 23 21 22 57 5 10 21
SlGABA -T1RNAi
SlGABA -T2RNAi
SlGABA -T3RNAi

Fig. 4

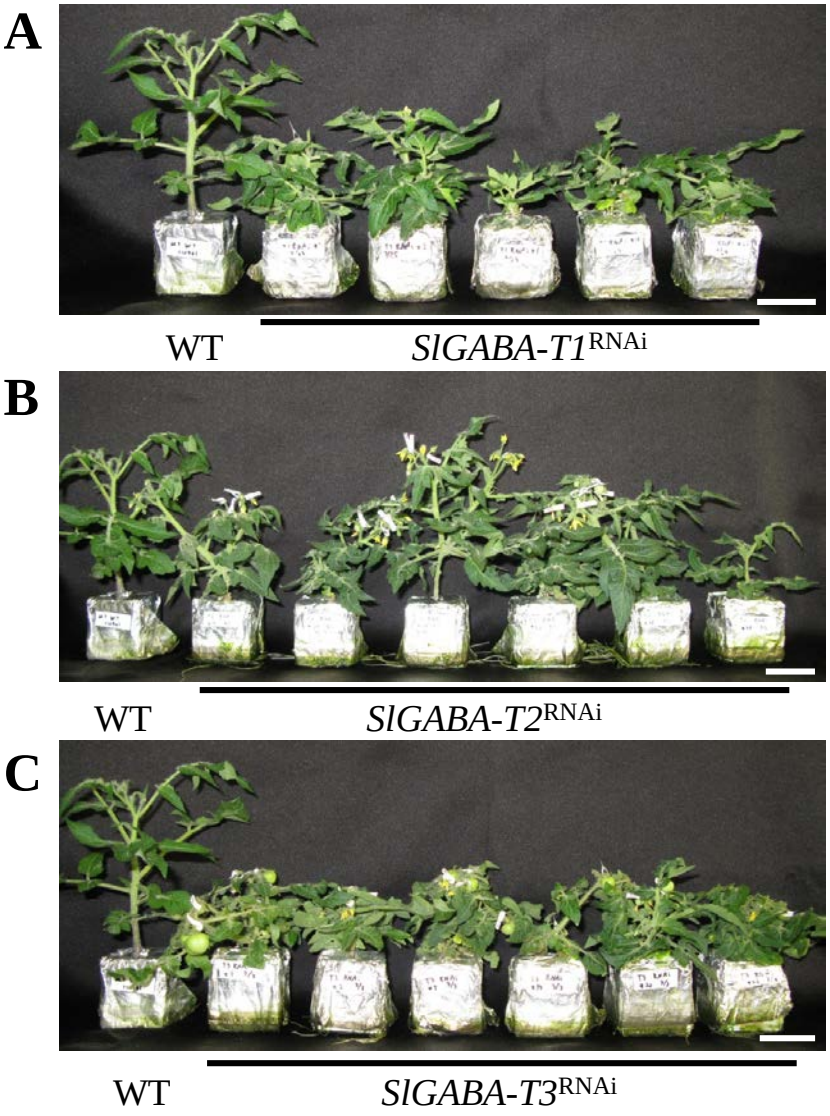
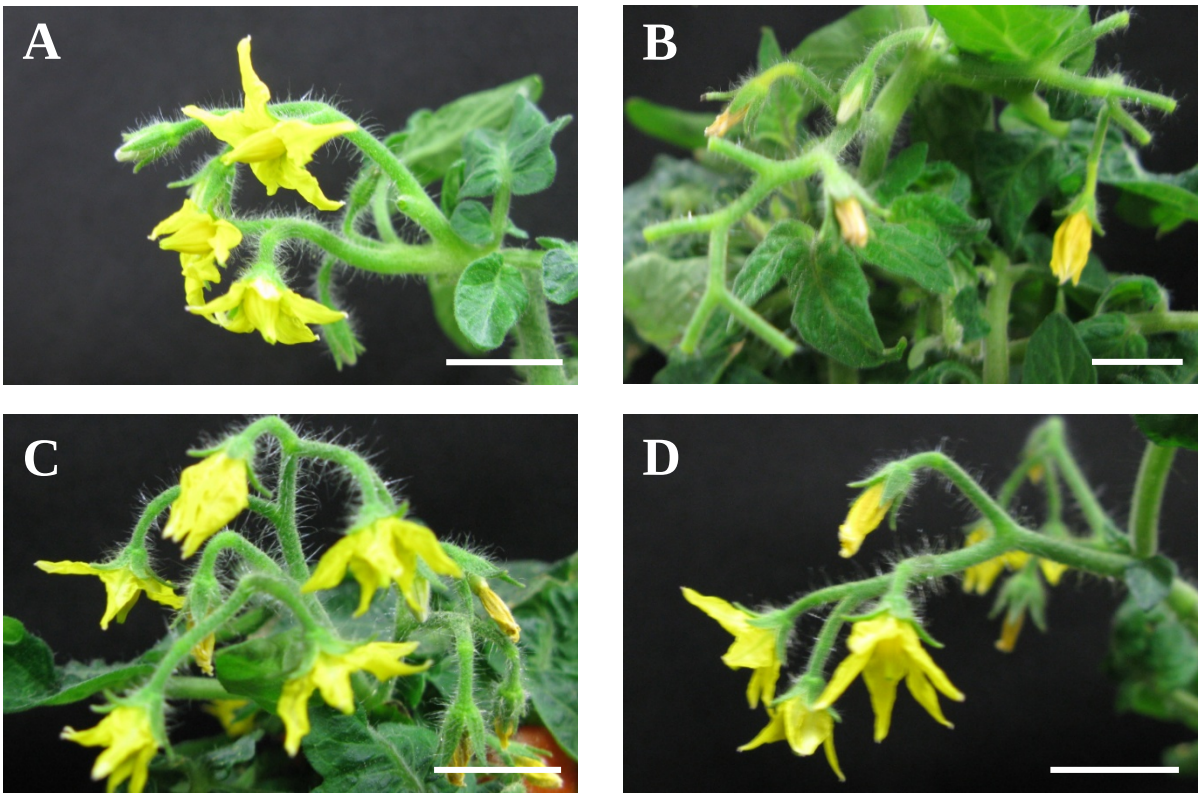

A
WT
SlGABA-T1RNAi
B
WT
SlGABA-T2RNAi
C
WT
SlGABA-T3RNAi
D
Plant height (cm)
15
10
5
0
WT
1
2
10
23
28
21
22
42
48
57
2
5
10
20
21
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig. 5


A
B
C
D

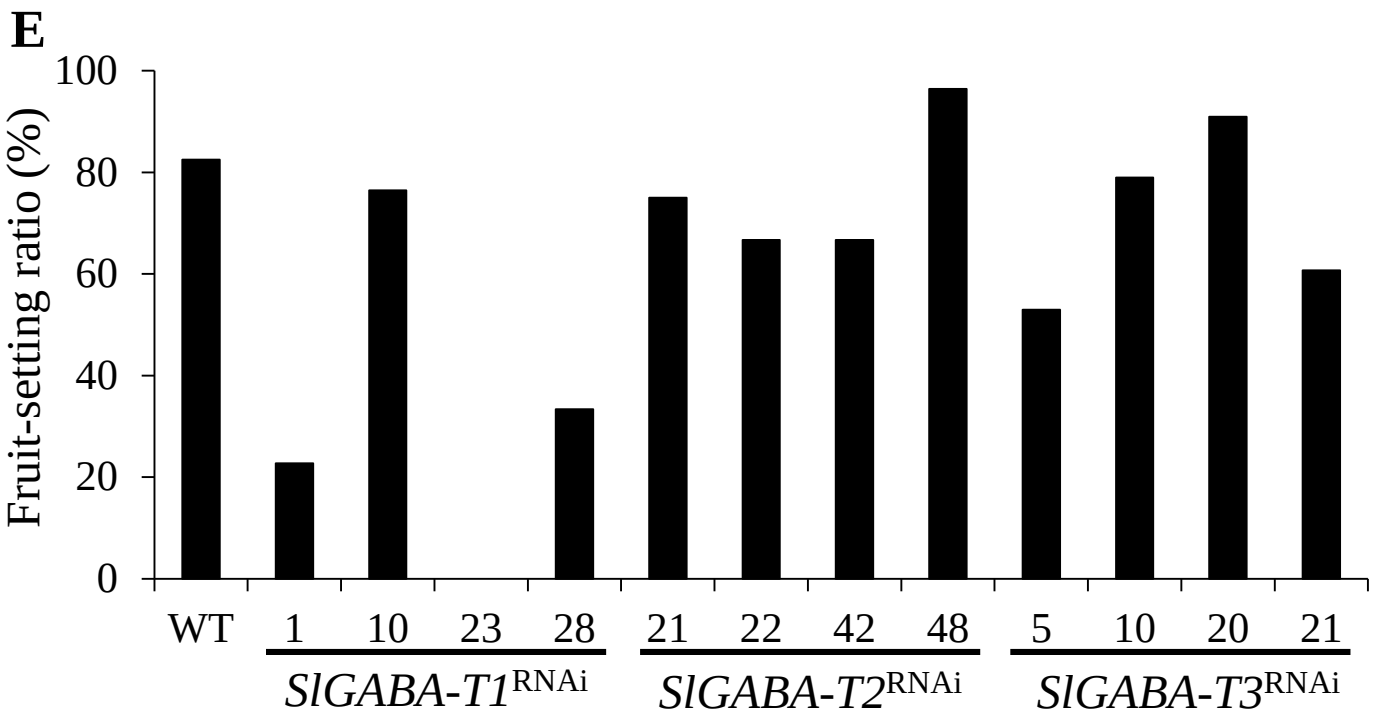

E
Fruit-setting ratio (%)
100
80
60
40
20
0
WT
1
10
23
28
21
22
42
48
5
10
20
21
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig. 6

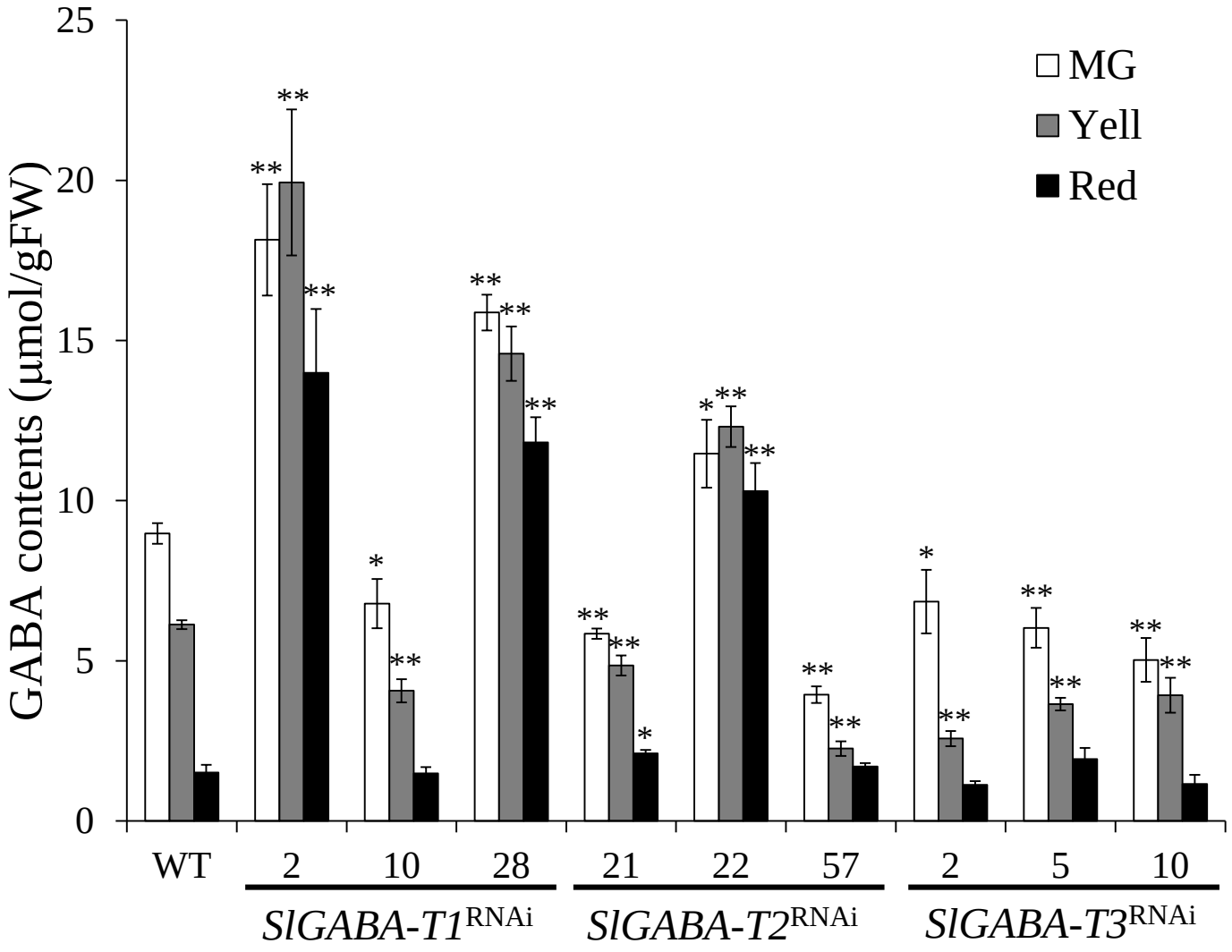

GABA contents (μmol/gFW)
25
20
15
10
5
0
MG
Yell
Red
WT
2
10
28
21
22
57
2
5
10
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig.7

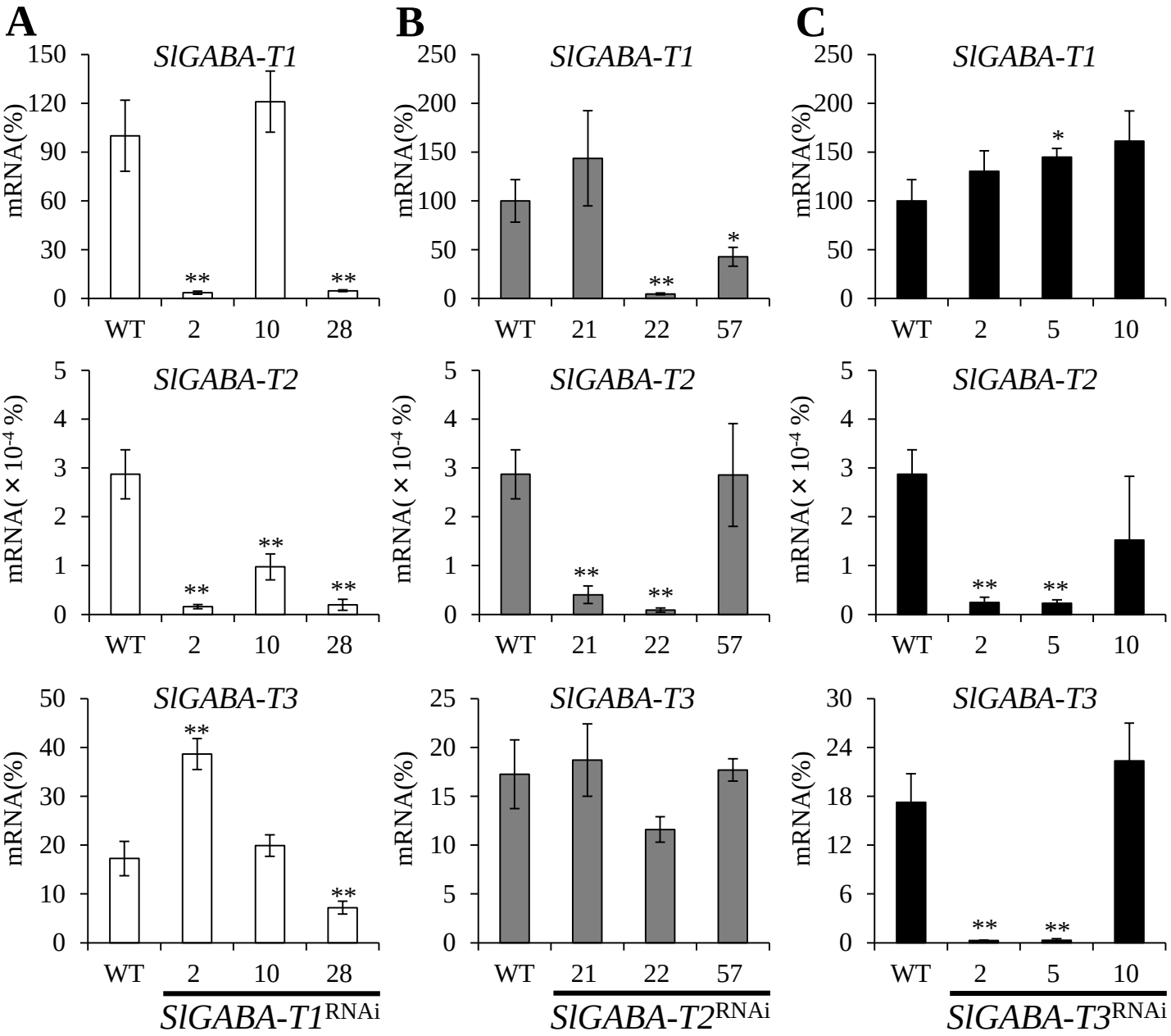

A
B
C
SlGABA-T1
SlGABA-T2
SlGABA-T3
mRNA(%)
mRNA(× 10-4 %)
WT
2
10
28
21
22
57
5
**
*
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig. 8

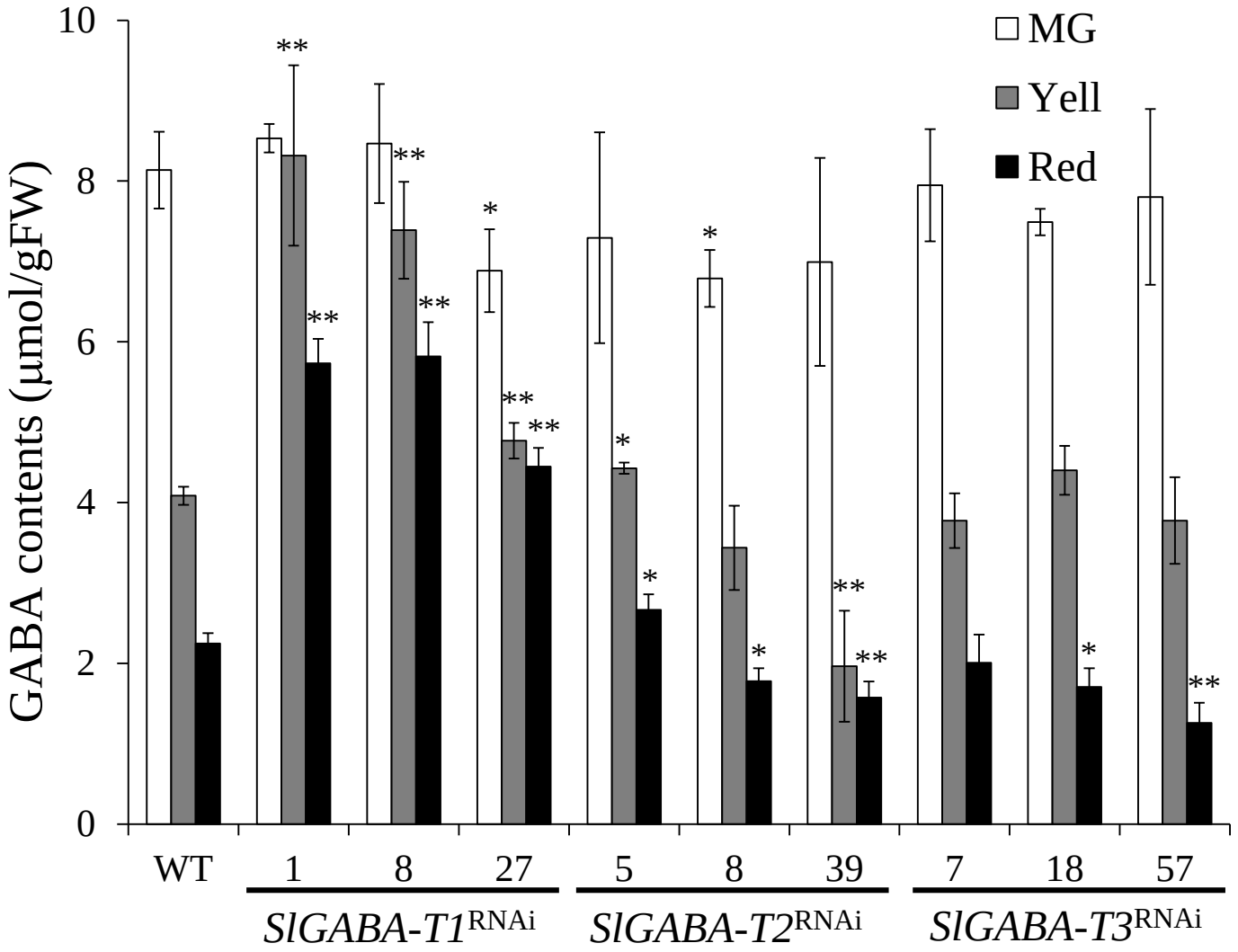

10
8
6
4
2
0
GABA contents (μmol/gFW)
MG
Yell
Red
WT
1
8
27
5
8
39
7
18
57
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig.9

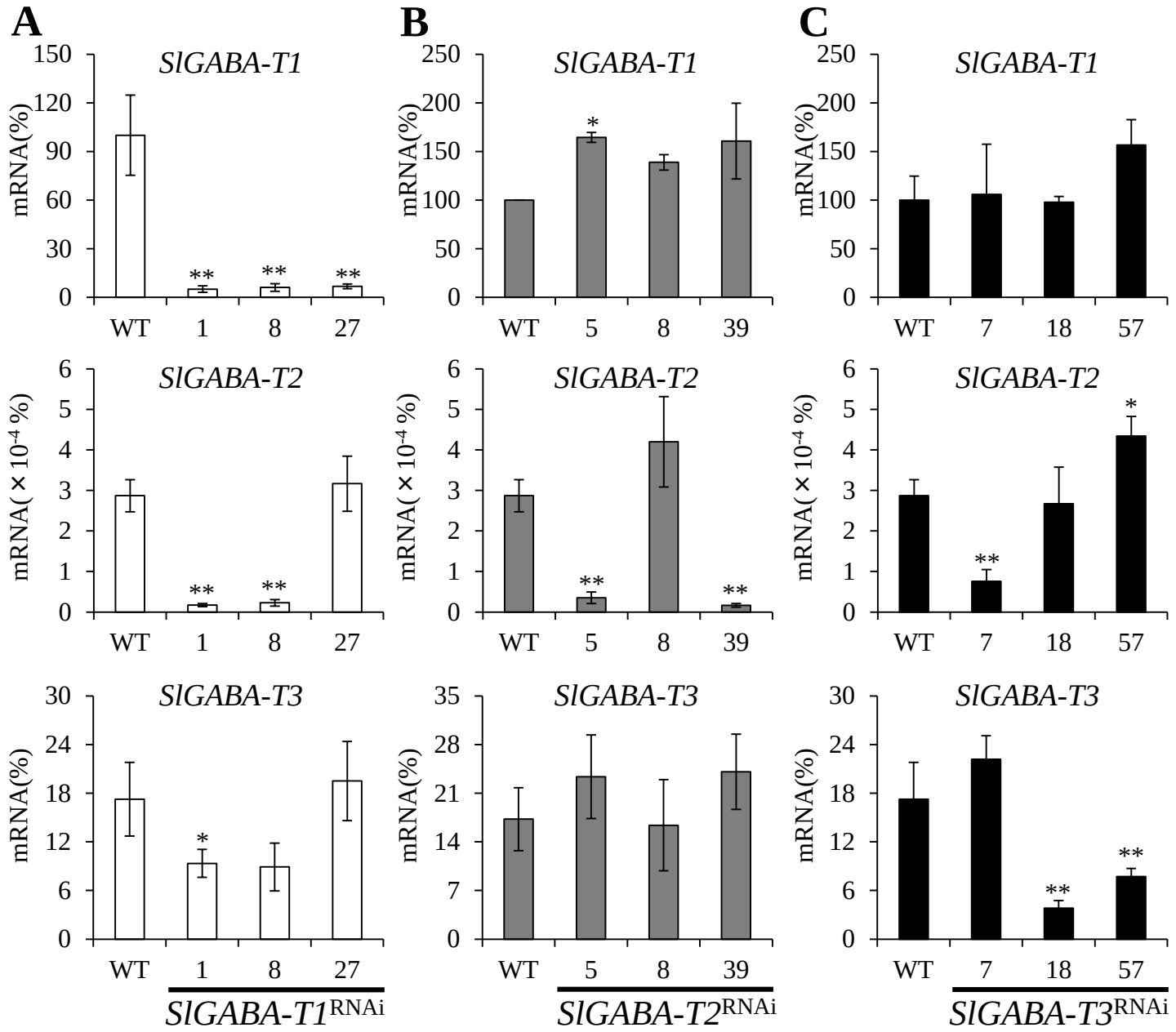
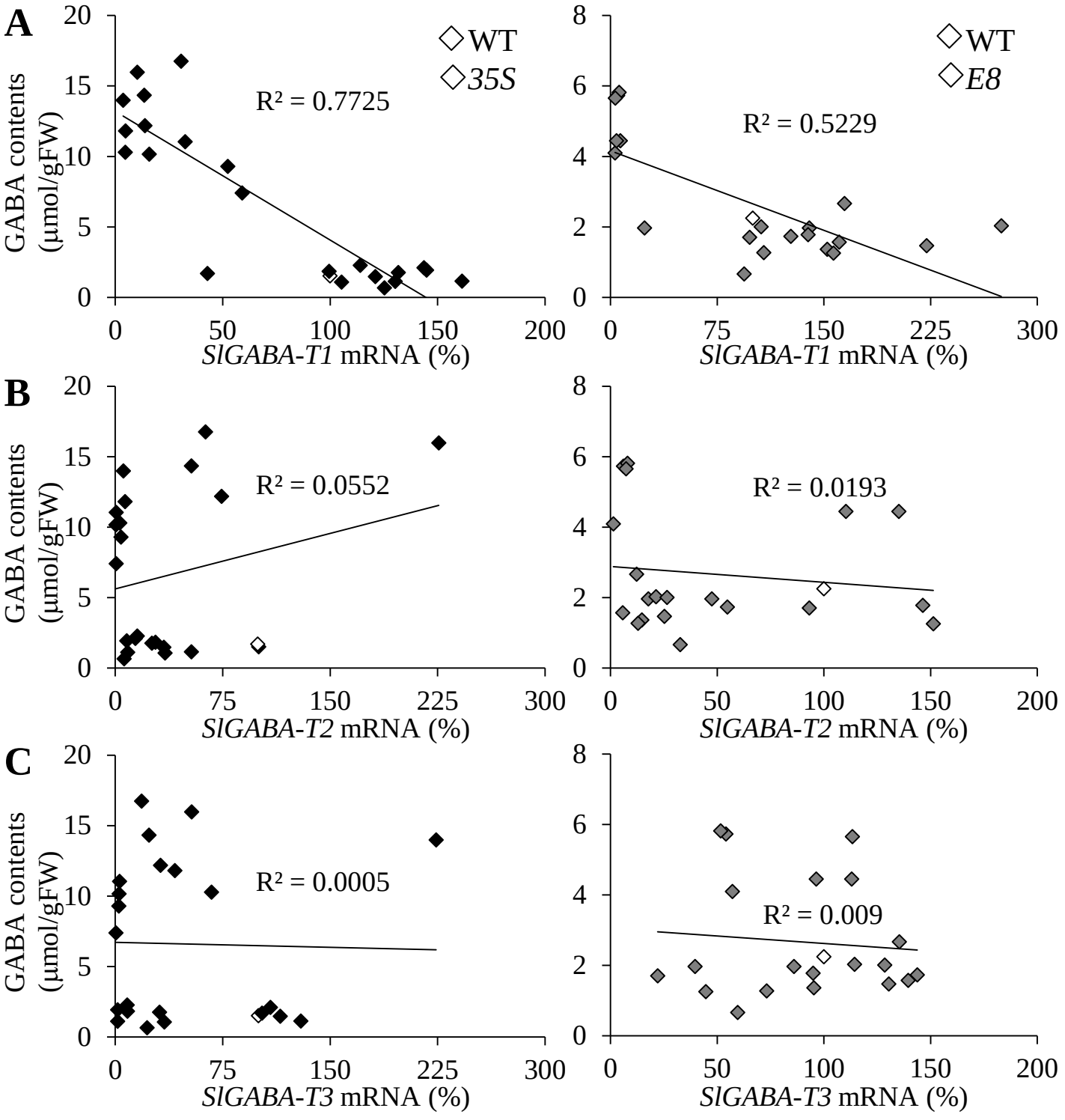

A
B
C
SlGABA-T1
SlGABA-T2
SlGABA-T3
mRNA(%)
mRNA(×10-4 %)
WT
1
8
27
5
39
7
18
57
SlGABA-T1RNAi
SlGABA-T2RNAi
SlGABA-T3RNAi

Fig. 10


A
GABA contents (μmol/gFW)
WT
35S
R² = 0.7725
SlGABA-T1 mRNA (%)
WT
E8
R² = 0.5229
SlGABA-T1 mRNA (%)
B
GABA contents (μmol/gFW)
R² = 0.0552
SlGABA-T2 mRNA (%)
R² = 0.0193
SlGABA-T2 mRNA (%)
C
GABA contents (μmol/gFW)
R² = 0.0005
SlGABA-T3 mRNA (%)
R² = 0.009
SlGABA-T3 mRNA (%)